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Magnesium and Anion Requirements of *rodB* Mutants of *Bacillus subtilis*

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rodB mutants of *Bacillus subtilis* have been found to require several hundredfold more Mg^{2+} in a minimal growth medium than the wild type to achieve rapid growth. In the presence of all concentrations of Cl^-, the organisms grow as deformed cocci, but with 10 mM Mg^{2+} and Br^-, I^-, or NO_3^- present they grow as rods. The morphology is then directly under the control of the concentration of both Mg^{2+} and anion. Originally, it was found that L-glutamic acid in the medium brought about the change from deformed spheres to rods. This amino acid will similarly function at a much lower concentration when the higher concentrations of Mg^{2+} and Cl^- are also present. At a constant concentration of L-glutamate, the morphology can be controlled by varying the Mg^{2+} concentration. In the presence of Mg^{2+} and I^-, the morphological change is temperature sensitive. At 30 C rods are formed and at 42 C deformed cocci are formed. The requirement of a *rodB* mutant for a high concentration of magnesium and the round morphology have been shown to be most probably due to a single mutation.

Three groups of Rod⁻ mutants of *Bacillus subtilis* have been distinguished by genetic and biochemical investigation (9). Both the temperature-sensitive *rodA* mutant (2) and the salt-sensitive *rodC* (9) showed a reduction in the proportion of teichoic acids in their walls when grown as deformed cocci under restrictive conditions. Phosphoglucomutase mutants of *B. licheniformis* and *B. subtilis* were coccal in shape when grown under phosphate limitation and had greatly reduced amounts of negatively charged polymers associated with their walls under these conditions (3). These observations suggested an important role for the negative polymers in maintaining the normal division and morphology of bacilli. However, the *rodB* mutants had the same total amounts of teichoic acid phosphorus in their walls, whether growing as cocci or rods (17). It has now been found that they have very high specific requirements for magnesium ions and for a limited range of anions for rapid growth. These *rodB* mutants were originally found to be corrected from cocci to rods by either high salt concentrations or by the addition of sodium L-glutamate to the growth medium (14). They also grew rapidly in the presence of this amino acid. It has now been observed that their morphology can be controlled by the concentration of Mg^{2+} in the growth medium, providing bromide, iodide, nitrate, or L-glutamate is also present.

MATERIALS AND METHODS

Microorganisms. The *rodB⁻* strain 104 (*rodB1 leuA8*), a transformant of the wild-type strain BD54 (*rod⁺ leuA8 metB5 ilvA3*) (9), was maintained on Luria agar at room temperature, being subcultured each week.

Media. In the first experiments the minimal medium of Sargent (19) was used. A low-phosphate medium termed TRM was then designed; to make it the following components, kept as sterile solutions, were mixed: 0.12 M Na^+-K^+ phosphate, pH 7.4, 10 ml; 0.2 M 3-(*N*-morpholino)propanesulfonic acid, 250 ml; 0.175 M tris(hydroxymethyl)aminomethane (Tris), 250 ml; 0.15 M $(NH_4)_2SO_4$, 10 ml; 10% (wt/vol) glucose, 50 ml; 0.36 mM $FeSO_4 \cdot 7H_2O$, 1.0 ml; 0.4 mM $MnSO_4$, 1.0 ml; 1 M Na_2SO_4, 10 ml; 0.4 M K_2SO_4, 25 ml. For the mutant, sufficient sterile leucine solution to give 40 μg/ml was added, and for the wild type sufficient sterile L-leucine, L-isoleucine, and L-methionine solutions to give 40 μg/ml were added, together with the required volumes of $MgSO_4$, anion solutions, and water to make 1.0 liter. The pH of the medium was 7.4 and the individual solutions were sterilized by autoclaving. The concentrations of phosphate, Na^+, K^+, Fe^+, Mn^{2+}, and glucose were sufficient to allow exponential growth of both the mutant and the wild type to an extinction value at 675 nm of at least 0.5 to 0.6.

For solid media, TRM was used as above, except that per liter, 12.5 ml 0.2 M Tris-H_2SO_4 (pH 7.4) was used instead of Tris-MOPS medium. It was solidified with Davis New Zealand agar powder at a final concentration of 1.5% (wt/vol). At such a concentration this agar contains approximately 0.013 mM

Mg^{2+} (R. F. Rosenberger, unpublished data).

T-S medium (containing 0.5% sodium glutamate) was as described by Karamata and Gross (8). SA agar was LS medium described by Karamata et al. (9) with 0.1% (wt/vol) Casamino Acids (Difco) and 0.5% (wt/vol) glucose plus required amino acids at 40 μg/ml. BHIB consisted of brain heart infusion broth plus 0.5% yeast extract (both from Difco).

Inoculation and cultural conditions. A loopful of each culture from the plate was inoculated into 5 ml of warm minimal medium containing 10 mM $MgSO_4$ and 15 mM NH_4Cl in a 50-ml flask and incubated with shaking at 35 C for 8 h. Dilutions of this culture from 1:1,000 to 1:100,000 were then made into prewarmed minimal media supplemented with appropriate concentrations of Mg^{2+} and anions contained in 100-ml flasks. These cultures were shaken at 35 C overnight. The dilutions that were then growing exponentially (optical density at 675 nm between 0.05 and 0.4) were transferred to further medium of the same composition so that the initial optical densities of the experimental cultures were 0.01 to 0.03. These final cultures of 10 ml each were contained in 100-ml flasks with side arms attached and were incubated at 35 C with shaking.

Transduction. Stocks of PBS1 transducing phage were prepared essentially as described by Karamata and Gross (8), except that BHIB was used to grow the donor and recipient strains and that a good "producer" lysate of PBS1, i.e., one with good ability to produce other lysates with transducing activity, was used (this was kindly provided by P. J. Piggot). For transduction, recipient bacteria were grown overnight in BHIB and, if motile, 1.0 ml was mixed with 0.1 ml of a PBS1 transducing lysate, incubated for 30 min at 35 C with shaking, centrifuged down, resuspended in Spizizen minimal medium (20) plus 0.5% (wt/vol) glucose (8) or TRM plus 0.5% (wt/vol) glucose, and plated on selective agar. Motility, necessary for PBS1 adsorption, was usually achieved by diluting the culture or by adding glucose if the overnight culture itself was not motile.

Scoring of phenotypes of recombinants and of revertants. The magnesium requirement was tested by spotting bacteria suspended in buffer plus 0.5% glucose (see Table 7) onto TRM plates containing no added Mg^{2+}, Cl^-, or sodium glutamate. The wild type (Mag^+) grows on these plates, whereas the *rodB* mutant 104 fails to do so.

The morphology was scored by spotting buffer suspensions of bacteria onto SA agar plates, allowing these to grow overnight, and observing the morphology of buffer suspensions using a Watson phase-contrast microscope. SA medium gave the most pronounced Rod^- (round) morphology of strain 104, whereas the wild type was Rod^+.

Measurements of cell dimensions. Samples from exponentially growing cultures were diluted into 5% formaldehyde solution and stored overnight at 0 to 4 C. The next day the lengths of the axes of the cells, as nearly at right angles to each other as possible, were measured. A Watson split-image eye piece with a total magnification of 1,500 was used. The preparations were examined wet on the slides and under a cover slip.

RESULTS

The *rodB* group of mutants had always grown well on the solidified SA agar on which they were isolated (15, 16) or in a variety of minimal liquid media containing glucose and sodium L-glutamate. In the absence of the glutamate, however, growth in either the original minimal medium (14, 16), in Spizizen salts medium (20), or in the medium designed by Sargent (19) was slow and variable, and very long lags often occurred after inoculation. In Spizizen medium without glutamate, growth frequently failed altogether. It was then observed that the addition of 10 to 15 mM NaCl to the Sargent (19) medium greatly improved growth. Growth usually occurred without a lag, and the doubling time was decreased from 200 to 300 min to 80 to 90 min. Comparison of NaCl, KCl, and NH_4Cl as additions showed that the anion, rather than the cation, was effective. Among the anions tested, bromide, iodide, and nitrate were equally effective, whereas sulphate, which was in any case already present in the Sargent medium, acetate, and formate were not effective. The wild-type BD54, of course, grew well on all the media without addition of anions. Since anion effects of the sort indicated here were unusual, particularly with bacteria, and because permeability studies with other systems, such as mitochondria, are usually more involved with the uptake of cations such as Na^+, K^+, NH_4^+, or Ca^{2+}, it was decided to examine more thoroughly not only the anion requirements of the mutant and wild type but also the cation requirements.

Magnesium requirements. The minimal medium described above was designed to eliminate the high-phosphate content of most other minimal media. Requirements for Na^+, K^+, PO_4^{3-}, and, of course, NH_4^+ could be readily shown but did not differ as between the mutant and the wild type. Examination of the effect of different concentrations of Mg^{2+}, however, immediately showed a very large difference. Maximum growth rates with a doubling time of about 60 min were achieved with a concentration of 0.1 mM by the wild type, whereas the mutant required 10 mM. This experiment was done with 15 mM KCl in all the media. A precise experiment was then done with various Mg^{2+} concentrations to determine the K_s (i.e., the concentration of a nutrient giving half-maximal growth rate [6, 10]) using the mutant and the wild type. The values obtained were 3 μM for the wild type and 1.2 mM for the mutant (Fig. 1). Both the mutant and BD54 were then tested in the absence and presence of KCl. The concentration of Mg^{2+} required by the mutant was between two and three orders of magnitude greater than for the wild type. With increasing chloride, the K_s for the mutant was reduced from 3 mM in its absence to 1.2 mM with a concentration of 15 mM. The anion had no effect on the K_s for Mg^{2+} of the wild type. The K_s value for Mg^{2+} of BD54 was also measured in Mg^{2+}-limited chemostat cultures (Fig. 1A). The slope of the line was different in the continuous culture from that obtained using batch cultures, and the K_s value was somewhat higher at 8 μM. Attempts to measure the K_s of the mutant in continuous culture were frustrated by reversion of the strain. Thus, it would seem possible that *rodB* mutants are lacking in transport of Mg^{2+} and that the uptake system is sensitive to halide ions. Mg^{2+} could not be replaced by Ca^{2+}, Sr^{2+}, Be^{2+}, or Ba^{2+}, the latter three cations being toxic.

Effect of Co^{2+}. Co^{2+}-resistant mutants of *Escherichia coli* are also deficient in Mg^{2+} transport (11, 12), as are those of *B. subtilis* (21). The toxicity of Co^{2+} has long been known to be antagonized by Mg^{2+} (1). Examination of the effects of Co^{2+} on the wild type and on the mutant showed that, indeed, the mutant was more resistant when the optimum requirements for Mg^{2+} and Cl^- were present. Whereas 20 to 50 μM Co^{2+} halved the growth rate of the wild type, the mutant was only slightly affected by 0.2 mM Co^{2+}. It must be pointed out, however, when the medium for both strains contained 3 mM Mg^{2+} and 5 mM Cl^-, little difference between them could be detected.

Anion requirements of the mutant in the presence of high Mg^{2+}. A wide variety of anions as their sodium, potassium, or ammonium salts was tested for their effects on the growth rate of the mutant in TRM minimal medium containing 10 mM Mg^{2+}. Three groups could be distinguished (Table 1); those that promoted the growth rate so that it was similar to that of the wild type, namely, Cl^-, Br^-, I^-, NO_3^-; those that caused some improvement, namely, CNS^-, IO_4, ClO_4^-; and a large group, including F^-, that had no effect or were toxic.

Morphological effects of inorganic anions. Thorough examination of combinations of concentrations of Mg^{2+} and Cl^- between 0 and 30 mM for each ion showed that the mutant always grew as deformed spheres. This was not so for the other anions effective in improving the growth rate. Table 2 shows the morphological effects of these anions as the ratio of the measured axes of the cells. Whereas at 1 mM Mg^{2+}, with all of the inorganic anions, this ratio was close to 1.0, with 10 mM Mg^{2+} and 15 mM

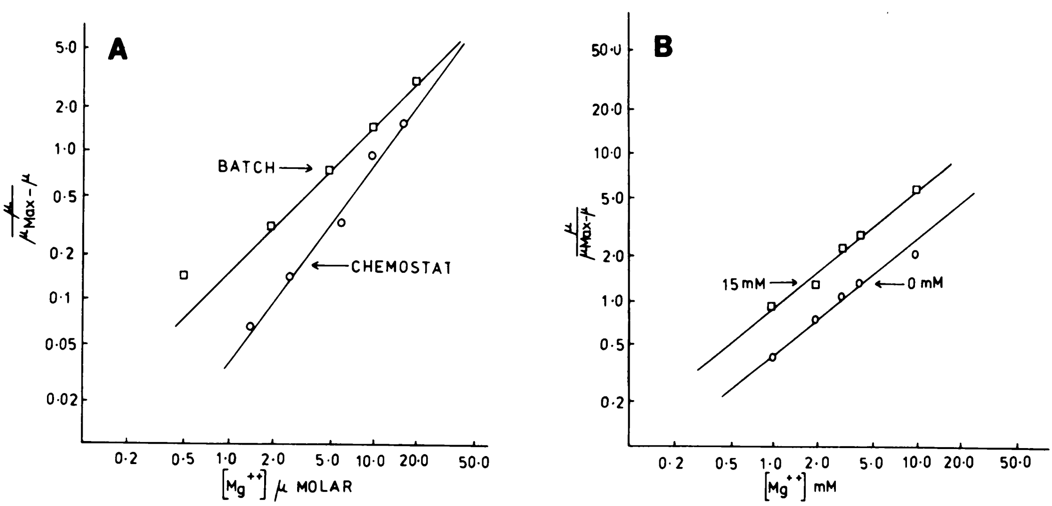

FIG. 1. *Double exponential plots of the effect of the concentrations of Mg^{2+} added to TRM minimal medium. (A) for BD54 growing in batch cultures containing either 0 or 15 mM Cl^- ions; (B) for the rodB mutant 104. The K_s values (i.e., the concentrations of Mg^{2+} necessary for half-maximal growth rates) are the concentrations of Mg^{2+} giving a value of 1.0 for the ratio $\mu/(\mu_{max} - \mu)$. The chemostat culture of BD54 was with a 2-liter volume and run at various dilution rates from 0.05 to 0.7 under Mg^{2+} limitation. The concentration of Mg^{2+} in samples of the supernatants of the cultures at these different dilution rates was measured by flame absorptiometry. The presence or absence of Cl^- ions (arrows) in the medium had no effect on the growth rates of BD54.*

TABLE 1. *Effect of different anions on the rate of growth in rodB strain 104 growing in TRM minimal medium containing sulfate*

Growth-effective anions			Noneffective anions		
Anion	Concn (mM)	Growth rate[a] (min)	Anion	Concn (mM)	Growth rate[a] (min)
SO_4^{2-} only		147[b]	$Fe(CN)_6^{4-}$	5	170
Cl^-	10	90	$S_2O_3^{2-}$	15	113
	15	75	CO_3^{2-}	15	140
Br^-	15	70	MO_4^{2-}	15	130
I^-	1	80	F^-	5	130
	10	75		15	130
NO_3^-	15	65			
CNS^-	5	90			
	10	85			
ClO_4^-	15	85			
IO_3^-	15	80			

[a] Doubling time in steady-state exponential growth.
[b] Some variation was found in the absence of effective ions. The result quoted is the mean value from five separate experiments. The range of values was 125 to 185 min.

TABLE 2. *Effect of anions on the morphogenic effect on rodB strain 104 of variable Mg^{2+} concentration*[a]

Mg^{2+} (mM)	L/D[b]				
	Cl^-	Br^-	I^-	NO_3^-	CNS^-
1		1.19	1.05	1.16	
10	1.14	2.15	2.02	1.98	1.57

[a] Concentration of the anions added to TRM was 15 mM, except for I^-, which was at 5 mM.
[b] L/D, Ratio of axes of the cells.

Br^-, I^-, and NO_3^-, it increased to almost 2.00. This value is similar to the value of 2.25 obtained for BD54 growing in 0.02 mM Mg^{2+} and 15 mM Cl^-, a value that rises to 2.9 with excess (for the wild type) of 10 mM Mg^{2+}. The anions that have some positive effect on growth of the organism, such as CNS^-, IO_3^-, and ClO_4^-, also showed a slight effect on the morphology, raising the ratio of the axes of the cells to 1.3 to 1.4. The relationship between the concentrations of Mg^{2+} and I^- was explored in detail. Figure 2 shows that, as the concentration of Mg^{2+} in the TRM medium containing 5 mM I^- was increased from 2 mM up to about 8 mM, the ratio of the axes of the cells increased from about 1.0 to about 2.0. The growth rates in these media were constant with a doubling time of 60 min, irrespective of the Mg^{2+} concentration present. The effect of increasing iodide concentration in the presence of a constant 10 mM Mg^{2+} is shown in Fig. 3. The cells became increasingly rodlike with added I^-, and with a concentration of 30 mM of the anion the ratio of the axes became 3.0. These effects on the morphology all involved a decrease in one axis of the cell and an increase in the other.

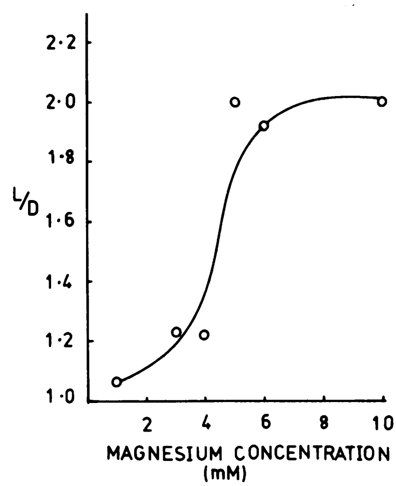

FIG. 2. *Effect of varying the Mg^{2+} concentration present in TRM minimal medium containing 5 mM I^- upon the axial ratios of cells of rodB strain 104. Cells from exponentially growing cultures were examined.*

There appears to be little evidence for competition between Cl^- and I^- in the morphological effect. The addition of up to 20 mM Cl^- to a medium containing 5 mM I^- and 10 mM Mg^{2+} may have very slightly depressed the ratio of the axes of the cells from 2.12 with no Cl^- present to 1.84 with 20 mM. The significance of such a small effect cannot be assessed.

Effects of L-glutamic acid. The original observation about *rodB* mutants was that in MMR liquid minimal medium (14) they re-

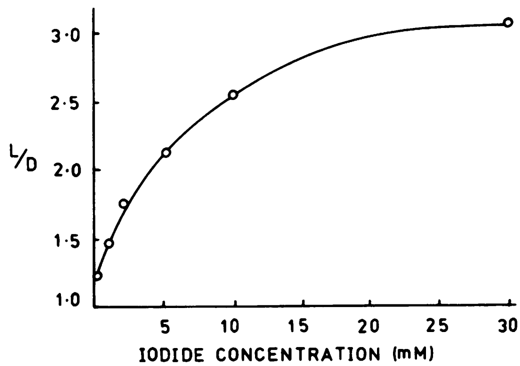

FIG. 3. *Effect of varying the I^- concentration in the presence of 10 mM Mg^{2+} in TRM medium upon the axial ratios of rodB strain 104. Cells from exponentially growing cultures were examined.*

quired a concentration of 8 mM sodium L-glutamate to convert them from deformed spheres to rods and that the principal role of 0.8 M NaCl, which also carried out this conversion on solid media containing traces of casein hydrolysate, was to facilitate the uptake of the amino acid when present in low concentrations. Morphological conversion occurred with 2 to 3 mM in the presence of 0.8 M NaCl. The present effects of very much lower concentrations of certain inorganic anions now raise the question of whether the glutamate may have acted simply as another anion. Reexamination of the full range of amino acids added to the TRM minimal medium in the presence of 10 mM Mg^{2+} and 15 mM Cl^- showed that, at a concentration of 0.25 mM, only glutamic acid was fully effective. Its metabolic relations arginine and proline partially converted the spherical forms to rods. Some activity was shown by threonine; otherwise, as before, all the other amino acids were completely inactive.

The effects of L-glutamate, Mg^{2+}, and Cl^- on the morphology in the TRM minimal medium are shown in Tables 3, 4, and 5. The concentrations of Cl^-, Mg^{2+}, and L-glutamate were all important. The change to cells that would be called rods on microscopic inspection was achieved by 0.06 mM glutamate if 10 mM Mg^{2+} and 15 mM Cl^- were present (Table 3). The cells, however, got somewhat longer and thinner with greater glutamate concentrations. With 6.0 mM the ratio of the axes reached the value of 2.8, compared with 1.9 at 0.12 mM (Table 4). The presence of glutamate had little influence on growth rate when 15 mM Cl^- was also present. When Cl^- was omitted, 0.12 mM glutamate increased the growth rate to the value obtained with 15 mM Cl^-. In the presence of L-glutamate and Cl^-, the morphology of the cells was completely controlled by the Mg^{2+} concentration (Table 5).

Temperature effects on morphology. Apart from a temperature sensitivity in the growth of the original Rod^- mutant (Rod 4) which failed to grow at 45 C, no evidence was found for temperature sensitivity. This temperature sensitivity was lost when the *rodB* mutation was transformed into BD54. When, however, cultures of the *rodB*$^-$ strain 104, growing in TRM at either 30 or 35 C in the presence of 10 mM Mg^{2+} and 5 mM I^- as rods, were switched to 45 C they changed to the characteristic spherical shapes. At the same time, the growth rate of the culture fell from having a doubling time of 50 min to one of 70 min. Both the mor-

TABLE 3. *Concentration of sodium L-glutamate required for the morphological transition of rodB strain 104*

Ion concentration (mM)		L-Glutamate concn[a] (mM)	Growth rate[b] (h)
Mg^{2+}	Cl^-		
1.0	16	1-3	1.3
5.0	15	0.6	1.1
10	15	0.06	1.1
10	0	>3.0	1.3

[a] Sodium L-glutamate concentration required for growth as rods.
[b] Doubling time in exponential growth.

TABLE 4. *Effect of 10 mM Mg^{2+} and 15 mM Cl^- upon the morphology of rodB strain 104*[a]

Cl^- concn (mM)	L-Glutamate concn (mM)	L/D	Growth rate[b]
0	0	1.14	3.1
15	0	1.10	1.3
0	0.12	1.19	1.3
15	0.12	1.90	1.2
0	6.0	1.89	1.2
15	6.0	2.82	

[a] L/D, Length/diameter of cells.
[b] Doubling time in exponential growth.

TABLE 5. *Effect upon the morphology of rodB strain 104 of varying the Mg^{2+} in TRM medium in the presence of 0.27 mM sodium L-glutamate*

Mg^{2+} concn (mM)	Length (L) (μm)	Diam (D) (μm)	L/D[a]
1	1.86	1.73	1.08
3	2.05	1.52	1.36
5	2.08	1.28	1.64
7	2.12	1.19	1.78
10	2.25	1.08	2.10

[a] L/D, Ratio of the lengths of the axes of the cells.

phological change and that in the growth rate were fully reversible. When the growth temperature was changed back to 30 C, the growth rate accelerated (Fig. 4) and rods reappeared. This temperature-sensitive change was not observed when 5 mM I^- was substituted by 15 mM Cl^-.

Genetics of strain 104. The *rodB1* mutation was isolated originally in *B. subtilis* 168 *trp* after treatment with the mutagen nitrosoguanidine (15, 16). Since this mutagen is known to produce multiple mutations (4), it was important to determine whether a single mutation was the cause of the two observable mutant phenotypes, i.e., high magnesium requirement (Mag$^-$) and round morphology (Rod$^-$). The multiple mutations caused by nitrosoguanidine have been shown to be mostly very closely linked (4) and, thus, the transfer of the Mag$^-$ and Rod$^-$ phenotypes by transformation (9) using saturating levels of deoxyribonucleic acid (congression [13]) did not rule out the possibility of two closely linked mutations being the cause of the two mutant phenotypes.

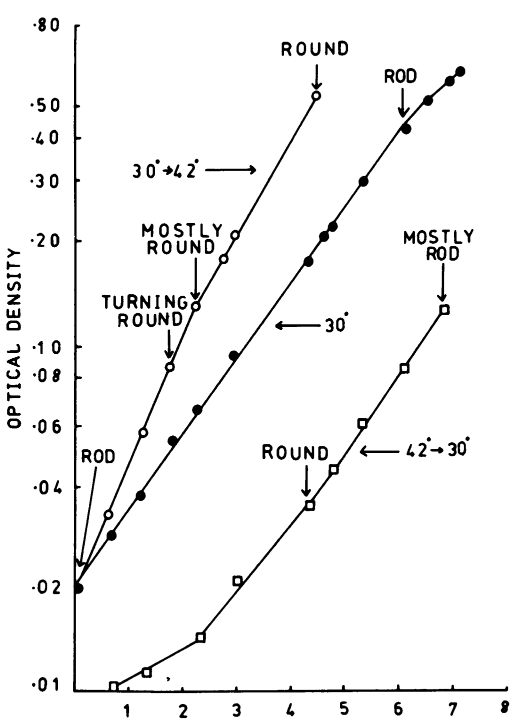

FIG. 4. *Effect of changing the growth temperature from 30 to 42 C upon the growth rate and morphology of rodB strain 104 growing in TRM medium containing 10 mM Mg^{2+} and 5 mM I^-. Symbols:* ●, *constantly at 30 C;* ○, *switched from 30 to 42 C;* □, *switched from 42 to 30 C.*

To answer this question, recombinants and revertants of strain 104 were isolated and studied. The generalized transducing phage PBS1 was grown on *B. subtilis* 168 *trp* (*trpC2 rod*$^+$), and the resulting phage lysate was used to transduce strain 104 (*rodB1 leuA8*) to select Leu$^+$ recombinants. *leuA* is known to be linked to *rodB* in PBS1 transduction (9). These transductants were scored for their Rod and Mag phenotypes. The results of three of these crosses are shown in Table 6; they indicate that, among 750 Leu$^+$ transductants of strain 104, only the donor (Rod$^+$ and Mag$^+$) and the recipient (Rod$^-$ Mag$^-$) phenotypes were observed. We were, thus, unable to separate the Rod$^-$ and Mag$^-$ phenotypes by recombination.

It could have been that recombinants were rare and were missed if the two postulated mutations were extremely closely linked. It was, therefore, decided to select Mag$^+$ revertants of strain 104 and to observe their morphology. Sixty-five independent Mag$^+$ revertants were selected on TRM plates lacking added Mg^{2+}, Cl^-, or sodium glutamate; of these, only one was Rod$^+$, the rest being Rod$^-$, a result which apparently suggested that Rod$^-$ and Mag$^-$ were due to separate mutations and could, thus, revert independently of one another. Seventy independent Mag$^+$ revertants were also selected on TRM plates lacking added Mg^{2+} or Cl^- but containing 0.5% sodium glutamate. None of these was Rod$^+$. To further test this, the Rod$^+$ Mag$^+$ revertant and one Rod$^-$ Mag$^+$ revertant were back-crossed into 104. To do this, a spontaneous

TABLE 6. *Phenotypes of Leu$^+$ transductants from a cross of strain 104 with phage PBS1 grown on 168 trp*[a]

Cross no.	No. of Leu$^+$ transductants	No. with phenotype of: Rod$^+$ Mag$^+$	Rod$^-$ Mag$^-$	% with *rodB-leuA* linkage
1	237	206	31	87
2	226	103	123	46
3	287	217	70	76

[a] Phage PBS1 grown on strain 168 *trp* (*leu*$^+$ *rod*$^+$) was used to transduce strain 104 (*leuA8 rodB1*), and Leu$^+$ transductants were selected, in the case of cross 1 on T-S plates and in crosses 2 and 3 on TRM plates containing 3 mM Mg^{2+}, 30 mM Cl^-, and 0.5% sodium glutamate. The transductants were purified by single-colony isolation and patched onto the same type of selective plates. A small portion of growth was suspended in 1 ml of Spizizen minimal medium or TRM (both plus 0.5% glucose), and this was spotted onto the appropriate plates (see text) to test for the Rod and Mag phenotypes. No Rod$^-$ Mag$^+$ or Rod$^+$ Mag$^-$ recombinants were observed from any of the crosses.

Leu$^+$ revertant of each strain was selected; these strains had reverted at the *leuA* locus since PBS1 lysates of these strains could co-transduce *leuA* and *pheA*, which are known to be linked using PBS1 transduction (9). Phage PBS1 grown on these Leu$^+$ strains was used to transduce strain 104 to Leu$^+$. With the Rod$^+$ Mag$^+$ strain as donor, of 59 Leu$^+$ transductants of strain 104, 45 were Rod$^+$ Mag$^+$ and 14 Rod$^-$ Mag$^-$, indicating a linkage of 76% between *rodB* and *leuA* and, thus, strongly suggesting that the site of the reversion to Rod$^+$ and Mag$^+$ was at or close to the *rodB* locus. On the other hand, with a Rod$^-$ Mag$^+$ revertant as donor, all 100 Leu$^+$ transductants of strain 104 were still Mag$^-$ (and Rod$^-$). Thus, the site of the Mag$^+$ reversion in this strain did not appear to map at the *rodB* locus, i.e., since it was not linked to *leuA*.

In fact, this latter revertant did not have the full Mag$^+$ phenotype of the wild type (unpublished data), and the reversion was, therefore, presumed to have occurred at some other locus affecting Mg^{2+} uptake. Revertants to Mag$^+$ occurred very frequently and there may, therefore, be a number of such loci affecting Mg^{2+} uptake. The Rod$^-$ Mag$^+$ strain appeared to be changed to a Rod$^+$ morphology by concentrations of Cl^- and sodium glutamate not affecting the morphology of 104 (Table 7).

We, therefore, can present no satisfactory evidence by reversion or recombination studies that the Rod$^-$ and Mag$^-$ phenotypes of strain 104 are due to more than one mutation.

DISCUSSION

Deficiency in the amounts of negatively charged polymers, such as the teichoic and teichuronic acids in walls of *rod* and other mutants of *B. subtilis*, has been shown to be correlated with abnormalities of division and morphology. It has been speculated (5) that one of the functions of these polymers is to hold a supply of divalent cations available to facilitate the action of membrane-bound biosynthetic enzymes. Some evidence for this hypothesis has been produced for the membrane-associated lipoteichoic acid in *B. licheniformis* (7). This possibility adds interest to the present findings. The *B. subtilis rodB* strain 104, which has approximately normal amounts of teichoic acids in its walls even when growing as a deformed coccus, has a greatly increased specific requirement for Mg^{2+} in the medium to give rapid growth. Genetic evidence supports the view that the requirement of the mutant for high concentrations of magnesium and the Rod$^-$ morphology are due to a single mutation. The divalent cation is only effective if anions, such as Cl^-, Br^-, I^-, NO_3^-, or glutamate, are also present. The effect of Cl^- is to alter the apparent K_s for Mg^{2+}, which may be a reflection of an altered K_m for Mg^{2+} transport, although precise uptake studies have not yet been undertaken. The halide anions, other than F^- and Cl^-, as well as NO_3^-, have a second effect in the presence of adequate supplies of Mg^{2+}, namely, to correct division and morphological abnormalities so

TABLE 7. *Growth and morphology of wild-type B. subtilis, the RodB$^-$ mutant 104, and two types of Rod$^+$ revertants of 104*[a]

Strain	Growth[b] and morphology							
	− − −[c]	− + −	+ + −	+ − −	+ − +	− − +	− + +	+ + +
104 (Rod$^-$ Mag$^-$)	−	+ Round	+ Mostly round	+ Round	+ Mostly rod	−	+ Rod	+ Rod
104 2A (Rod$^-$ Mag$^+$)	+ Round	+ Rod	+ Rod	+ Mostly round	+ Rod	+ Rod	+ Rod	+ Rod
104 3A (Rod$^+$ Mag$^+$)	+ Rod	+ Rod	+ Rod	NT	NT	+ Rod	+ Rod	+ Rod
BD54 (Rod$^+$ Mag$^+$)	+ Rod	+ Rod	+ Rod	+ Rod	+ Rod	+ Rod	+ Rod	+ Rod

[a] The strains were grown overnight as patches on TRM plates containing 3 mM Mg^{2+}, 30 mM Cl^-, and 0.5% (wt/vol) sodium glutamate. A small portion of growth was suspended in TRM liquid medium lacking added Mg^{2+}, Cl^-, or glutamate, and this was spotted onto agar plates containing TRM with combinations of added Mg^{2+}, Cl^-, and glutamate, respectively, as shown. The plates were incubated overnight at 35 C, and the bacteria were observed by phase-contrast microscopy for their morphology.

[b] +, Growth; −, no growth; NT, not tested.

[c] Presence (+) or absence (−) of 3 mM Mg^{2+}, 30 mM Cl^-, and 0.5% sodium glutamate, respectively.

that the mutant grows as a rod: at a constant concentration of the anions, the degree of correction is related to the Mg^{2+} concentration. It is tempting to suppose the primary effect of the anions is to ensure the right supply of Mg^{2+} at the right place in the cell. Formally, nevertheless, there is no proof that the morphological effects are not due to special effects of the anions per se, but the great chemical differences between them make this idea somewhat unattractive. The effects of L-glutamate were already known and had been regarded as specific for either the amino acid or its amide. Preliminary comparison (unpublished work) of the metabolism and incorporation of the amino acid into proteins and mucopeptide had not shown any differences between the mutant and the wild type. The present observations emphasise the possibility that glutamate may simply serve in the presence of Mg^{2+} as another effective anion, like I^-, Br^-, or NO_3^-. It may be noted that L-aspartate in the presence of Mg^{2+} behaves like Cl^- in allowing rapid growth of the deformed coccal-shaped cells without correcting the morphology.

Part of the Mg^{2+} content of bacterial cells is in the walls, where it is bound by weak secondary linkages which are probably ionic since it can be removed by treating the cells with dilute NaCl solutions. Another part is firmly associated with the ribosomes. Smaller amounts are likely to be present in the membranes and as free Mg^{2+} in the cytoplasm, where it would be available to activate those "soluble" enzymes that need it. A very tentative suggestion for the effects of the anions is that they act in two ways: (i) to alter the K_m of the Mg^{2+} transport system in the mutants; (ii) for those that also alter the morphology, to shift the balance of Mg^{2+} concentrations in the various compartments of the cell. No evidence (18) was found for alterations in the rate of mucopeptide synthesis when *rodB* 104 was changed from a coccus to a rod by the addition of L-glutamate to the medium. It, therefore, seems unlikely that the ion effects described here will be due to activation or inactivation of wall biosynthetic enzymes. Different amounts of Mg^{2+} in the cytoplasmic membrane, however, could alter the lateral mobilities of proteins. If the distribution of active biosynthetic enzymes were altered, this might provide a reasonable explanation for the morphological changes.

The cause of the high magnesium requirement of the *rodB* strain 104 may be hypothesized to be a defect in the Mg^{2+} transport system. This is currently under investigation. Since there were large numbers of revertants that were still Rod^- on agar plates containing low concentrations of Mg^{2+} and because in the case of the one strain tested the site of the reversion was not linked to *rodB*, it appears that there may also be a number of other loci, besides *rodB*, the mutation of which affects magnesium requirement.

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